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13. ABSTRACT *(Maximum 200 words)*

Casualties resulting from the French and German incursions into Russia during the winters of the 19th and 20th centuries, respectively exemplify cold weather as a strategic element of war. From Valley Forge to the Chosin Reservoir, the U.S. Army has been made aware of the influence of cold weather conditions on soldier health. In times of relative peace, cold weather injuries for troops under training or deployment exceeded 500 cases for the winter of 1999/2000. While respectively cases of frostbite, immersion foot and chilblains occur more frequently than hypothermia; this more lethal form of cold-related insult represents a soldier injury in need of remedy. However, as in the days of the American Revolution, the doctrine of increasing insulation or evacuation to warmer environments remains the primary approach to guard against hypothermic mortality. Perhaps understanding the influences of hypothermia on body systems supporting resistance to stress and trauma may reveal strategies to lessen the possibility of a lethal consequence following hypothermia. The contribution of the reticuloendothelial system (RES) to survival following traumatic stress is well documented. This is especially true for physiological insults involving vascular shock, to include that associated with heatstroke. This impact of the RES is mediated, in part by its clearance of vascular debris to support blood vessel patency. The present study examined the effect of rat whole body hypothermia and hypothermia with rewarming on RES blood clearance and PU by RES organs. Findings revealed hypothermia significantly decreased RES clearance as a result of reduced liver particulate uptake. Following active rewarming, this decrement in RES function remained. Further study is recommended to determine if modulations in RES clearance capacity alters the outcome of hypothermia. Such studies may establish RES fitness as an important concept to support reductions in cold-induced mortality.

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BACKGROUND

The considerable influence of the reticuloendothelial system (RES) on recovery following trauma and/or vascular shock (1-5,7-11,15,16,20) supports a concept that RES fitness is an important survival factor following physiological stress associated with significant tissue injury. Findings from USARIEM suggest this concept is relevant in heat stress survival, as well. For example, enhanced RES blood clearance capacity correlates with an improved heat stress survival rate in rats (7). In addition, the non-specific opsonin, plasma fibronectin (PF) that mediates the phagocytic process of RES particulate uptake (PU) leading to blood clearance is a marker for heat stress survival in rats (8). Finally, PF level is elevated by activities that result in improved physical fitness or thermotolerance in humans (3). The relevance of such human PF elevations as a marker for thermotolerance is supported by the absence of PF elevations seen in passive acclimation with seasonal change in which physiological adaptation factors improve, but thermotolerance is not achieved (4). The present study evaluated the effects of whole body hypothermia on RES blood clearance and PU to estimate the potential of RES fitness as a protective paradigm in this form of environmental stress. A rat model (14) was employed to define the capacity of RES organs (lung, liver and spleen) to clear from the blood a fluorescent marker during whole body hypothermia and following active rewarming (REW).

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EXECUTIVE SUMMARY

Casualties resulting from the French and German incursions into Russia during the winters of the 19th and 20th centuries, respectively exemplify cold weather as a strategic element of war. From Valley Forge to the Chosin Reservoir, the U.S. Army has been made well aware of the influence of cold weather conditions on soldier health. In times of relative peace, cold weather injuries for troops under training or deployment exceeded 500 cases for the winter of 1999/2000. While respectively cases of frostbite, immersion foot and chilblains occur more frequently than hypothermia, this more lethal form of cold-related insult represents a soldier injury in need of remedy. However, as in the days of the American Revolution, the doctrine of increasing insulation or evacuation to warmer environments remains the primary approach to guard against hypothermic mortality. Perhaps understanding the influences of hypothermia on body systems supporting resistance to stress and trauma may reveal strategies to lessen the possibility of a lethal consequence following hypothermia. The contribution of the RES to survival following traumatic stress is well documented. This is especially true for physiological insults involving vascular shock, to include that associated with heatstroke. This impact of the RES is mediated, in part by its clearance of vascular debris to support blood vessel patency. The present study examined the effect of rat whole body hypothermia and hypothermia with REW on RES blood clearance and PU by RES organs. Findings revealed hypothermia significantly decreased RES clearance as a result of reduced liver PU. Following active REW, this decrement in RES function remained. Further study is recommended to determine if modulations in RES clearance capacity alters the outcome of hypothermia. Such studies may establish RES fitness as an important concept to support reductions in cold-induced mortality.

INTRODUCTION

The RES is comprised of the endothelium that forms the interior lining of all blood vessels, as well as the specialized phagocytic cells residing in the lung, liver and spleen (1). This system is known to influence survival following vascular shock and/or tissue injury induced by hemorrhage (9-11,20), endotoxin (10,15,16,20), epinephrine (11), trauma (2,20), acceleration (15), tourniquet occlusion (16) or heat stress (3-5,7,8). Furthermore, shock susceptibility is increased by RES blockade (2,7,10,16,20). As early as the middle of the 20th century, the RES was hypothesized to be a common pathway for both the pathogenesis of and host resistance to shock (9,20). In part, this contribution to shock and/or tissue injury survival is mediate by RES phagocytic clearance of particulate debris to sustain blood vessel patency (1). In this regard, Kupffer cells, the specialized phagocytic entities of the liver are responsible for the majority of PU by the RES (1,5). Since RES function contributes to survival following tissue damage induced by a broad spectrum of physiological insults, it was of interest to determine if hypothermia impacted RES function. The present study defined the effects of hypothermia and hypothermia followed by REW on RES blood clearance and PU. The findings identified cold-induced RES decrements, to support study of RES fitness enhancement as an intervention to improve the outcome following hypothermia.

METHODS

Male Sprague-Dawley rats weighing between 375 to 425 g were employed. Using aseptic technique, anesthetized (intraperitoneal Nembutal, 45 mg/kg; intramuscular atropine, 200 µg) animals had a cannula surgically implanted within the jugular vein. Each animal was then given an intramuscular injection of Polyflex® ampicillin and allowed to recover for a minimum of 5 days prior to the start of experimentation.

Nine groups of 12 rats each were studied. Control groups comprised rats at a core temperature (T_c) of 37°C in the absence (AA) or presence of pentobarbital anesthesia (A; intraperitoneal Nembutal, 35 mg/kg) over 3 (A3h37°C) or 4 (A4h37°C) hours. Hypothermic groups were composed of anesthetized rats cooled to a T_c of 30, 25 or 20°C (A30°C, A25°C or A20°C, respectively). REW groups had anesthetized rats that were initially cooled to a T_c of 30, 25 or 20°C and then actively REW (A30°C~REW, A25°C~REW or A20°C~REW, respectively).

A rectal probe placed 6 cm beyond the rat's anal sphincter monitored T_c. Cooling or cooling followed by active REW was conducted as previously described (14). Briefly, anesthetized rats were placed within the loops of a copper coil. Water from a water bath initially set at 15°C was circulated through the copper coil. Over the first 15 min of water circulation, the water bath temperature was reduced until it reached 5°C. The circulating water was maintained at this temperature until a desired rat T_c was obtained. Rats remained at the desired T_c for 1h by manipulation of water flow rate and/or temperature. Following an hour of hypothermia, if active REW was to be employed, the temperature of the circulating water was gradually increased (1°C/min) until it reached

37°C. To prevent overshoot of normal body temperature, active REW was ceased when a T_c of $35\,^{\circ}$C was reached. At this point, rats continued to passively REW.

For groups in the absence (AA37°C) or presence of anesthesia (A3h37°C and A4h37°C), all rats received, via the jugular cannula fluorescein-labeled microspheres (FM; 1μ; 2×10^{10}/kg; Polysciences, Warrington, PA). The cannula was flushed with saline (0.5 ml) to removed residual FM and at 5 mins post FM injection a 0.5 ml blood sample collected. At 15 mins post FM injection, all rats were deeply anesthetized (methoxyflurane) and exsanguinated via cardiac puncture. Spleen, lung and liver (median lobe) samples were then collected and processed along with the 5 and 15 min blood samples in 15% KOH, as previously described (5,6). Similar procedures were followed for the other rat groups, with the FM injection occurring at 1 h of hypothermia or after REW.

As previously described (5,6), FM and phycoerythrin-coated bead (PE; 1μ; internal standard; Polysciences, Warrington, PA) stocks were placed in an ultrasonic water bath for 30 mins. Dilutions of the FM (1×10^6) and PE (1×10^5 and 1×10^6) were made after each stock was vortex mixed for 10 sec. A 1:1 dilution of the diluted FM (1×10^6) and PE (1×10^6) was used to align the beam and tune an Epics XL (Coulter, Corp., Miami FL) flow cytometer fitted with a 15 mW argon laser, which provided excitation at 488nm. A bitmap region was drawn below the fluorescent intensity of FM and PE. The flow cytometer was programmed to reject data within this region to exclude the relatively non-fluorescent tissue debris found within the digestates. Bandpass filters of 530nm and 585nm were used to collect FM and PE emissions, respectively. Digestates (0.5 ml) were placed (30 min) in the ultrasonic water bath, vortex mixed (10 sec) and then diluted 1:1 in either 1×10^5 PE (blood) or 1×10^6 PE (spleen, liver and lung) and analyzed on the flow cytometer. FM numbers were related to aspiration volume, which was determined by the counts of the PE internal standard. FM numbers per volume were translated to numbers per gram of tissue found in the original digestate. This value (FM concentration) was then expressed as the percent of the total number of FM injected into the rats per gram of tissue (% injectate/g of tissue).

Comparisons of FM concentration were made using analysis of variance over all experimental conditions (presence or absence of anesthesia-normothermic, anesthesia-hypothermic and anesthesia-hypothermic with REW) for each tissue (blood and RES organs). With values expressed as means ± S.E., points of significance ($p<0.05$) were determined using Tukey post hoc analysis.

RESULTS

Since this model employed anesthesia, the RES blood clearance and PU effects associated with differences in length of anesthesia during the various procedures to induce hypothermia or hypothermia with REW needed to be defined. The A30°C, A25°C and A30°C~REW groups required 3h, while the A20°C, A25°C~REW and A20°C~REW groups required ≥ 4h of anesthesia. Respectively, the A3h37°C and

A4h37°C groups served as the anesthesia controls for the two lengths of anesthesia employed.

As illustrated in Figures 1 and 2, there were no significant differences among the % injectate/g of blood at 5 or 15 min post FM injection for normothermic rats in the presence [A(3 or 4h)37°C] or absence (AA 37°C) of anesthesia. However, significantly higher levels of injectate remained in the blood samples collected 5 mins post FM injection in all hypothermic conditions (A30, 25, or 20°C) compared to normothermic rats in the presence or absence of anesthesia (Fig 1). Findings were similar for hypothermia followed by REW (A30°C~, 25°C~ or 20°C~REW), with the exception the A20°C~REW group value was significantly greater than the normothermic control in the absence, but not the presence of anesthesia. By 15 mins post FM injection only blood samples from the A20°C group still had significantly higher injectate levels when compared to normothermic controls in the presence or absence of anesthesia (Fig 2).

As noted for blood, no significant differences among the % injectate/g of spleen (Fig. 3) or lung (Fig. 4) values for normothermic rats in the presence or absence of anesthesia were recorded. Similarly, length of anesthesia, degree of hypothermia or REW after hypothermia had no significant influence on spleen values. Moreover, with the exception of the A20°C group, no condition of anesthesia length, hypothermia or REW significantly altered the % injectate/g of lung values compared to normothermic rats in the presence or absence of anesthesia (Fig. 4).

In general, anesthesia reduced PU by the liver (Fig 5). However, while there was a significant reduction associated with 3h [A(3h)37°C], this was not true for 4h [A(4h)37°C] of anesthesia, when comparisons were made to normothermic controls in the absence of anesthesia (AA37°C). Though, with the exception of A20°C~REW compared to A20°C, there were no significant differences among the % injectate/g of liver values for the hypothermia or hypothermia with REW conditions. However, such values were all significantly different than normothermic controls in the absence of anesthesia (AA37°C). This was not always true when comparisons were made to their appropriate anesthesia control, since only the A30°C and A20°C groups were significantly different from such controls [A(3 or 4h)37°C].

DISCUSSION

As recently reviewed, hypothermia and rewarming from hypothermia is associated with cardiac insufficiency that reflects a shock-like state (17). Shock induced by a variety of insults (2,9-11,15,16,20), to include hyperthermic shock (3,5,7,8) is known to influence RES function. However, a paucity of information is available in regards to hypothermic effects on the RES. The present study defined the influence of hypothermia and hypothermia with REW on RES blood clearance of FM and their PU by RES organs. It employed a model that permitted simultaneous induction of whole body hypothermia to multiple rats (14). This extended the use of the rat in defining hypothermic pathophysiological events, which have previously included studies on cardiac function (18), blood flow (19) and tissue extravasation (13).

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A reduced capacity to remove particulate from the blood stream is the hallmark for impaired RES clearance function (1). That blood particulate concentration at 5 mins post FM injection was significantly elevated for all hypothermic conditions (A30, 25 or 20°C) compared to normothermic controls in the absence of anesthesia (AA37°C) suggested RES impairment by whole body hypothermia (Fig. 1). Since differences remained significant when comparisons were made to anesthetized, normothermic controls [A(3 or 4h)37°C], the effects of anesthesia did not significantly contribute to this impairment. Thus, hypothermia clearly contributed to this phenomenon. With mild (Tc=30°C) or moderate (Tc=25°C) hypothermia followed by REW (A30°C~REW or 25°C~REW), a return to normalcy was not achieved, since blood particulate values were significantly greater not only to normothermic controls in the absence, but also the presence of anesthesia. However, values for extreme hypothermia (Tc=20°C) followed by REW (A20°C~REW) where not significantly different from normothermic controls in the presence of anesthesia. Though this indicated an anesthetic influence, it was not concluded RES clearance capacity had recovered with REW from extreme hypothermia, since mild or moderate hypothermia did not give evidence for such a recovery. Moreover, A20°C rats had significantly elevated blood particulate levels compared to all other conditions tested, even after an increase of clearance time to 15 min (Fig. 2). This was a further sign of the severity of RES impairment by extreme hypothermia, which suggested recovery with REW was an unlikely prospect.

Examination of PU by the major RES organs following hypothermia can identify the sites of RES clearance impairment. Changes in spleen PU by mild, moderate or extreme hypothermia and such degrees of hypothermia following by REW revealed no significant differences (Fig. 3). These findings likely reflected the relatively small size of this RES organ and its limited cellular phagocytic capacity. Moreover, the FM concentration was geared to the clearance capacity of the largest RES organ, the liver. Thus, under all conditions, sufficient particulate reached the spleen such that differences from normothermic controls were not distinguished. The same was true for the lung, with the exception A20°C rats had significantly higher particulate levels (Fig. 4). Tveita *et al.* report significant reductions in cardiac output (<10% of normothermic controls) by rats at a Tc=15°C (19). Thus, blood flow thru the lung is quite reduced in profoundly hypothermic animals. As such, the full bolus of FM administered via the jugular cannula resided within the lung an extended period of time, before it was disseminated to other areas of the body. Extended residency of highly concentrated FM would be a strong driving force for enhanced PU per phagocytic event. This would explain the significantly higher levels of particulate in the lungs of A20°C rats.

As expected, due to the liver's large size and specialized phagocytic function, the reduced liver PU during hypothermia and following REW (Fig. 5) was the major contributor to elevated blood particulate levels (Figs.1 and 2). All hypothermia and REW groups had significantly lower liver PU than non-anesthetized, normothermic controls. Factors such as blood flow (19), blood vessel permeability (13) and the presence of anesthesia may have influenced these liver findings. Of these, anesthesia would normally be extrinsic to naturally occurring hypothermia. Three hrs of anesthesia in normothermic animals significantly reduced liver PU, but by 4 hrs the effects of anesthesia had waned, such that significant differences were no longer apparent (Fig.

5). Mild, moderate and extreme hypothermic liver PU values were significantly lower than normothermic rats in the absence of anesthesia. This was true for mild and extreme, but not moderate liver PU value comparisons to anesthetized, normothermic controls. While this moderate hypothermic outcome remains an unexplained anomaly, because the mild and extreme conditions were significant when compared to anesthetized controls, reduced liver PU was largely considered the result of intrinsic hypothermic factors, rather than the extrinsic factor of anesthesia. In contrast, of the REW groups, only the moderate (A25°C~REW), but not the mild (A30°C~REW) or extreme (A20°C~REW) condition was significantly different to anesthetized, normothermic controls, which suggested effects of anesthesia may have appreciably contributed to the lowered liver PU values following REW. As such, recovery of liver PU following REW may not have been fully revealed due to the influences of anesthesia in the model. However, the fact 5 min blood particulate findings were significantly elevated compared to anesthetize, normothermic controls for the less severe REW conditions (A30°C~REW and A25°C~REW; Fig. 1) indicated recovery of liver PU was not fully achieved following REW.

A puzzling issue was the few significant differences in blood particulate level remaining at 15 min (Fig 2), even though significant liver PU differences at 15 min were quite apparent for mild, moderate and extreme hypothermia and REW (Fig 5). However, if each hypothermia and REW condition was individually compared to the normothermic controls in the presence or absence of anesthesia, rather than comparing all groups together as in Figure 2, then 15 min blood particulate values for all conditions were significantly different from such controls. Nevertheless, since when all groups were compared together at 5 min, blood particulate values were significantly different (Fig. 1), 5 rather than 15 min liver PU measurements would have improved the clarity of this study.

As previously mentioned, reduced blood flow likely augmented the lung PU in extreme hypothermic rats (A20°C; Fig. 4). However, this effect in the lung had an opposite result in the liver (A20°C; Fig 5), since elevated lung PU meant down stream organs, like the liver were exposed to a reduce FM concentration. Such findings illustrated an intrinsic impact of cold-induced blood flow changes on RES PU. Though another intrinsic factor, cold-induced increases in blood vessel permeability should augment the FM concentration in tissues, this did not appear to make a significant contribution, since in general such concentrations remained similar (Fig 3 and 4) or were significantly reduced (Fig. 5) compared to normothermic controls. If as shown for inflammatory mediators (12), gaps in the endothelium induced by cold did not exceed a diameter of 1μ, then perhaps the large size of the FM (1μ) employed in this study limited the effects of increased blood vessel permeability on the PU findings.
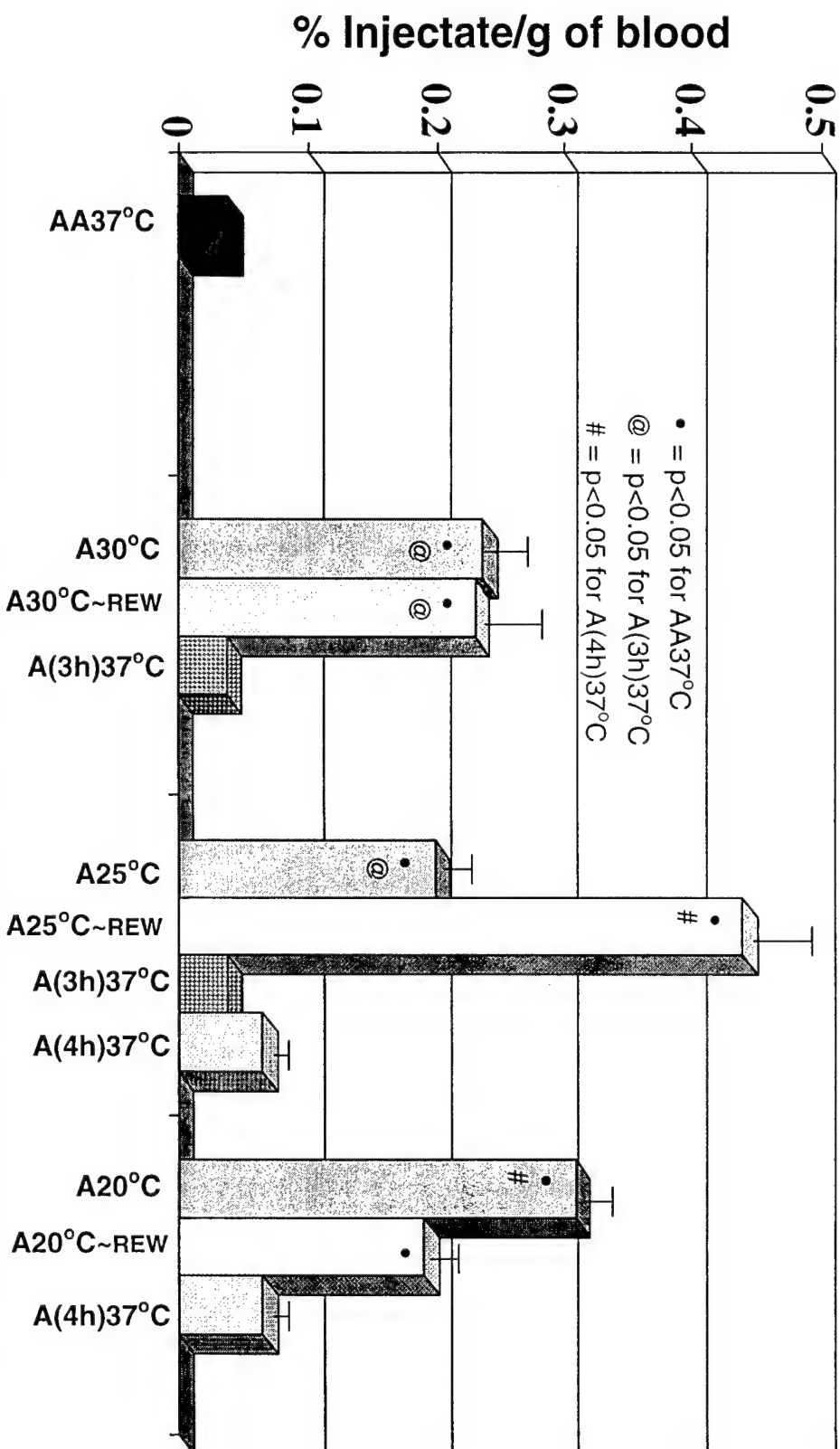
CONCLUSIONS

Hypothermia significantly impaired particulate blood clearance by the RES. Though somewhat clouded by anesthesia effects, REW did not result in recovery of this RES function. The major cause for reduced RES clearance was identified as the hypothermic effects on liver PU. Previously reported (19) reductions in cardiac output

and blood flow mediated by hypothermia likely contributed to the decreases in RES blood clearance and PU. Elevations in blood vessel permeability associated with hypothermia (13) may not have contributed significantly to the findings due to the large size of the vascular particulate employed in this study.
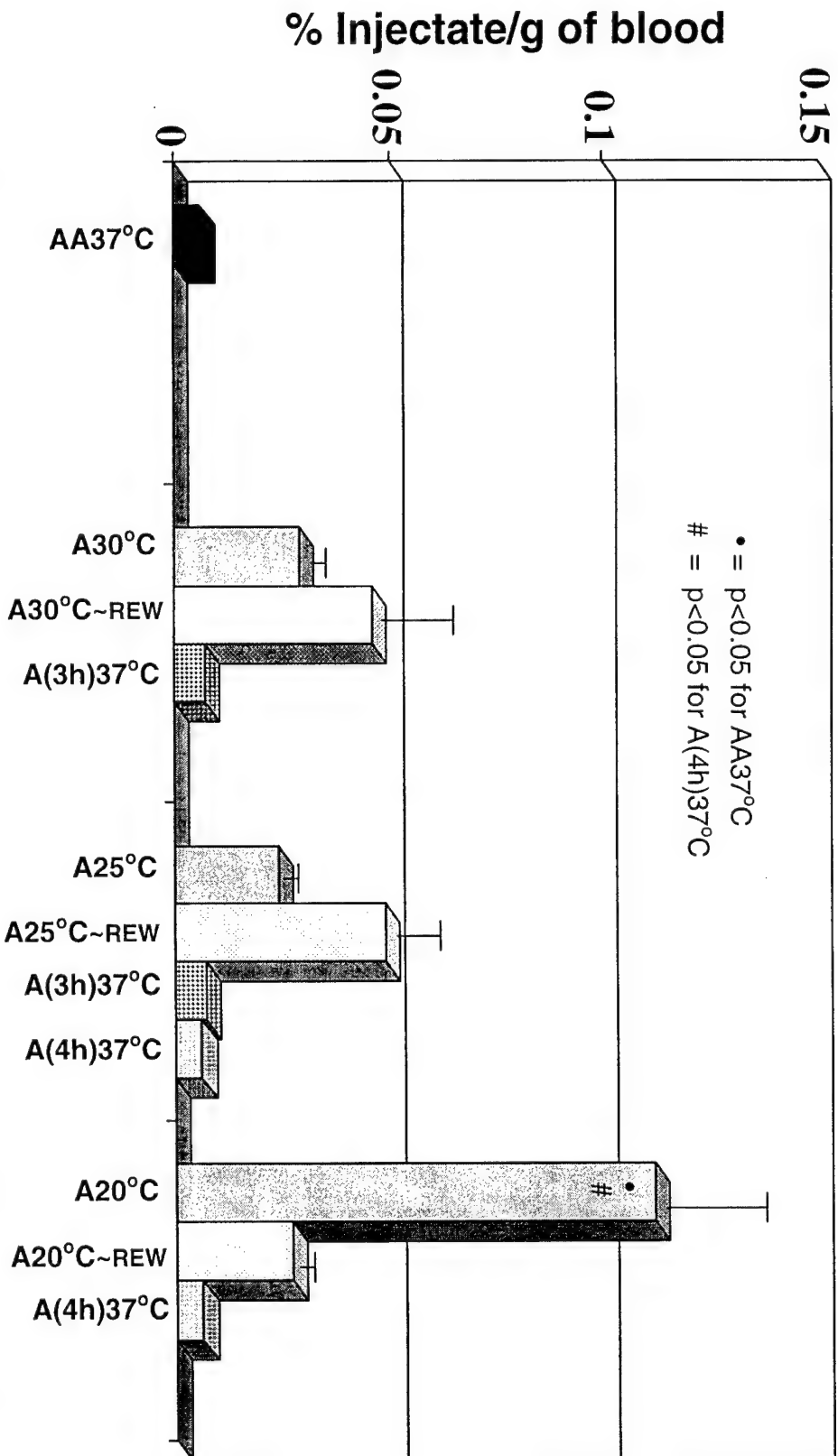
RECOMMENDATIONS

The RES is a common pathway for both the pathogenesis of and host resistance to shock. Hypothermia and REW from hypothermia are associated with a shock-like state and as the present study demonstrated impaired RES clearance function. Further study is recommended to define decrements in RES function following longer periods (i.e., 3 to 24h) of recovery post REW. This would assist identification of the lower limit of body temperature to which RES function does not normally recover. Knowledge of this lower limit could then be used to design experiments to determine if prior stimulation of RES function alters the outcome of hypothermic shock. Such studies may establish RES fitness as an important concept to support reductions in cold-induced mortality.

Figure 1. Comparison of blood particulate concentration (means±SE) 5 minutes post particulate injection among normothermic (37°C) absence of anesthesia (AA), anesthesia (A) hypothermic (A30, 25 or 20°C), anesthesia hypothermic with rewarming (~REW) and anesthesia (3 or 4h) normothermic rats*.

• = p<0.05 for AA37°C
@ = p<0.05 for A(3h)37°C
# = p<0.05 for A(4h)37°C

*The A25°C rats have two anesthesia normothermic controls, since to reach 25°C required 3, while rewarming from hypothermia required 4h of anesthesia.

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Figure 2. Comparison of blood particulate concentration (means±SE) 15 minutes post particulate injection among normothermic (37°C) absence of anesthesia (AA), anesthesia (A) hypothermic (A30, 25 or 20°C), anesthesia hypothermic with rewarming (~REW) and anesthesia (3 or 4h) normothermic rats*.
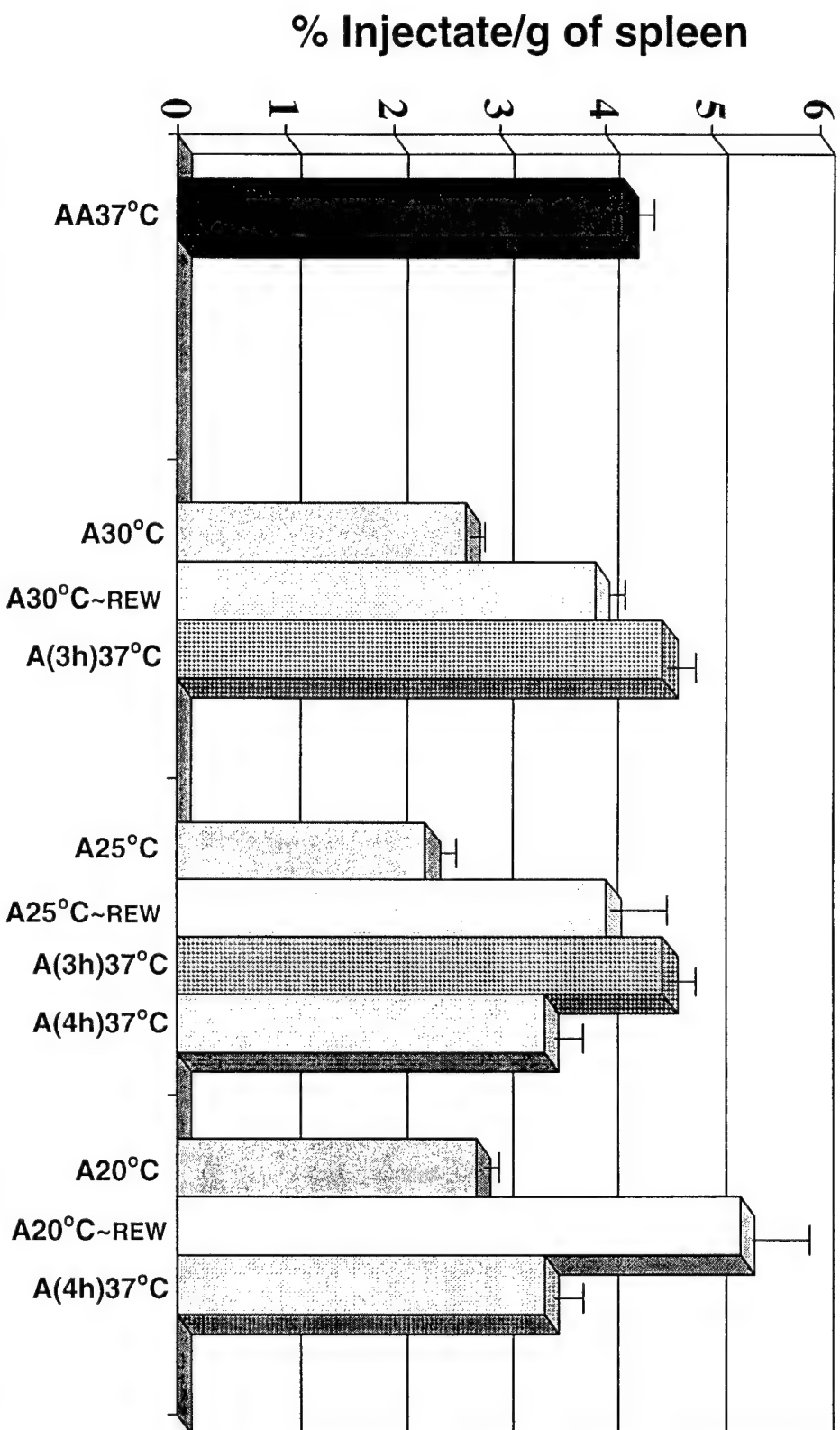
• = p<0.05 for AA37°C
= p<0.05 for A(4h)37°C

*The A25°C rats have two anesthesia normothermic controls, since to reach 25°C required 3, while rewarming from hypothermia required 4h of anesthesia.

9

% Injectate/g of spleen

Figure 3. Comparison of spleen particulate uptake (means±SE) among normothermic (37°C) absence of anesthesia (AA), anesthesia (A) hypothermic (A30, 25 or 20°C), anesthesia hypothermic with rewarming (~REW) and anesthesia (3 or 4h) normothermic rats*.
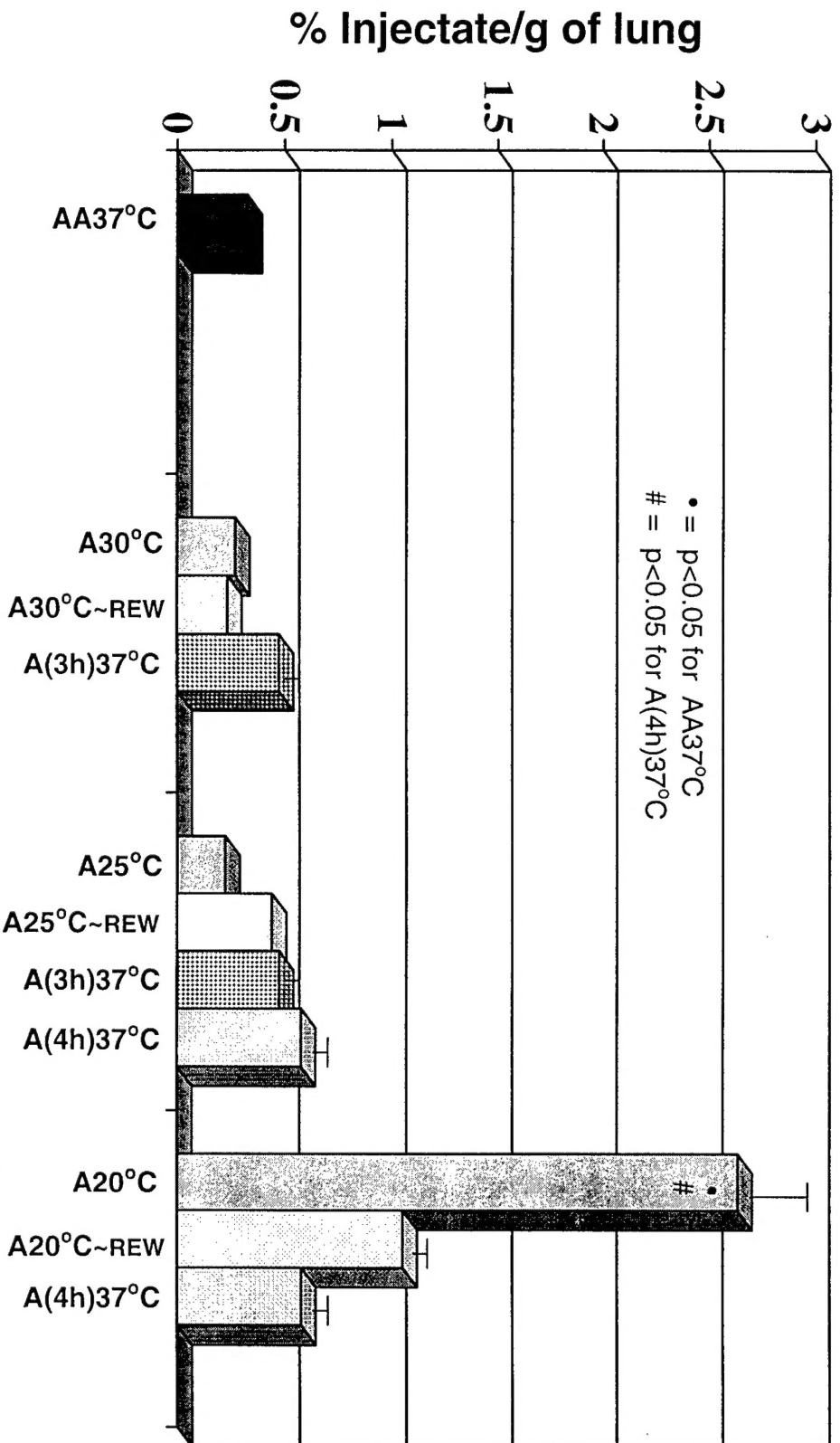
*The A25°C rats have two anesthesia normothermic controls, since to reach 25°C required 3, while rewarming from hypothermia required 4h of anesthesia.

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Figure 4. Comparison of lung particulate uptake (means±SE) among normothermic (37°C) absence of anesthesia (AA), anesthesia (A) hypothermic (A30, 25 or 20°C), anesthesia hypothermic with rewarming (~REW) and anesthesia (3 or 4h) normothermic rats*.

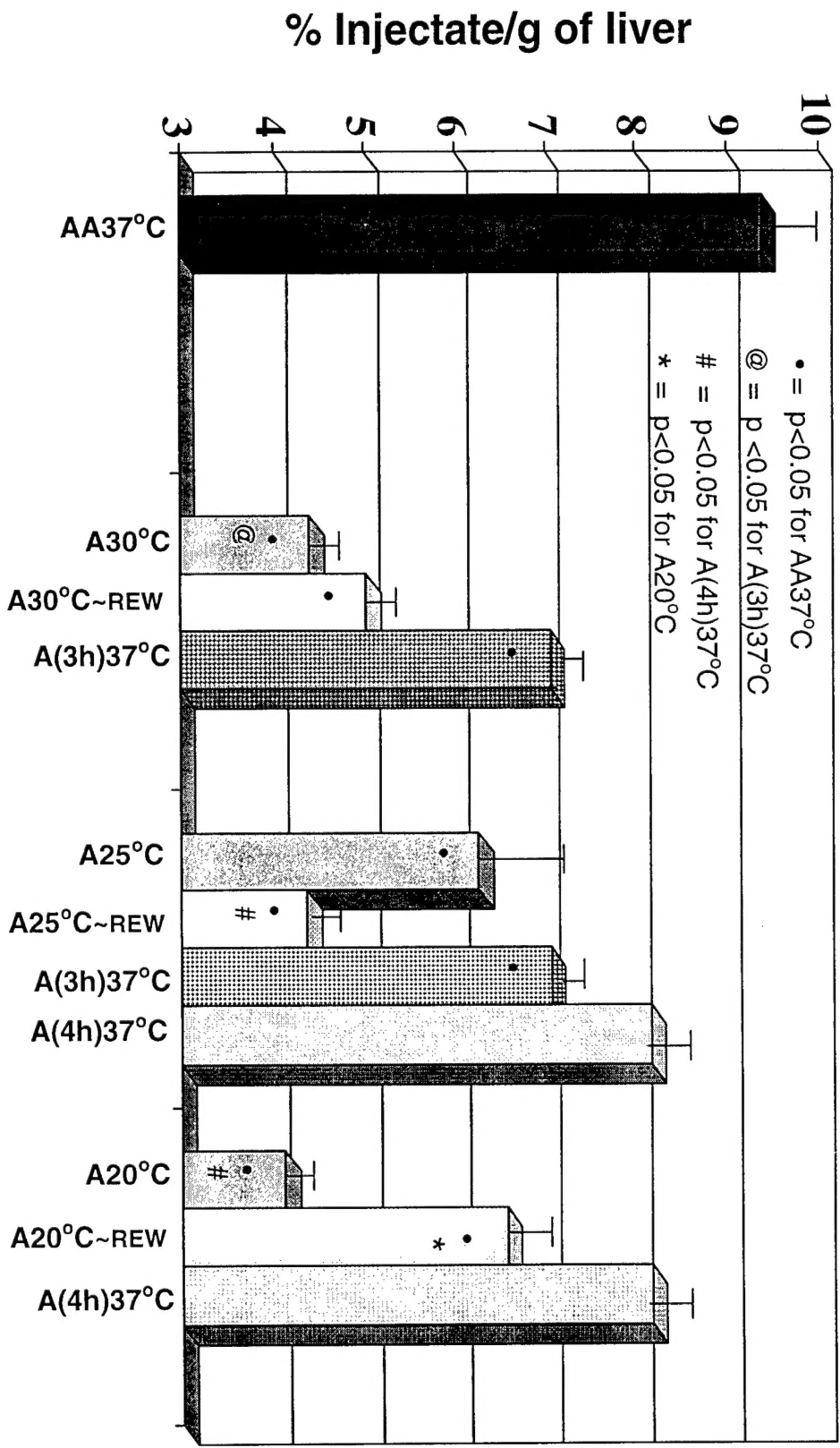• = p<0.05 for AA37°C
= p<0.05 for A(4h)37°C

*The A25°C rats have two anesthesia normothermic controls, since to reach 25°C required 3, while rewarming from hypothermia required 4h of anesthesia.

11

% Injectate/g of liver

Figure 5. Comparison of liver particulate uptake (means±SE) among normothermic (37°C) absence of anesthesia (AA), anesthesia (A) hypothermic (A30, 25 or 20°C), anesthesia hypothermic with rewarming (~REW) and anesthesia (3 or 4h) normothermic rats*.

Y-axis labels (bars): AA37°C, A30°C, A30°C~REW, A(3h)37°C, A25°C, A25°C~REW, A(3h)37°C, A(4h)37°C, A20°C, A20°C~REW, A(4h)37°C

X-axis: 3, 4, 5, 6, 7, 8, 9, 10

Legend:
- • = p<0.05 for AA37°C
- @ = p <0.05 for A(3h)37°C
- # = p<0.05 for A(4h)37°C
- * = p<0.05 for A20°C

*The A25°C rats have two anesthesia normothermic controls, since to reach 25°C required 3, while rewarming from hypothermia required 4h of anesthesia.

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